Figure S1

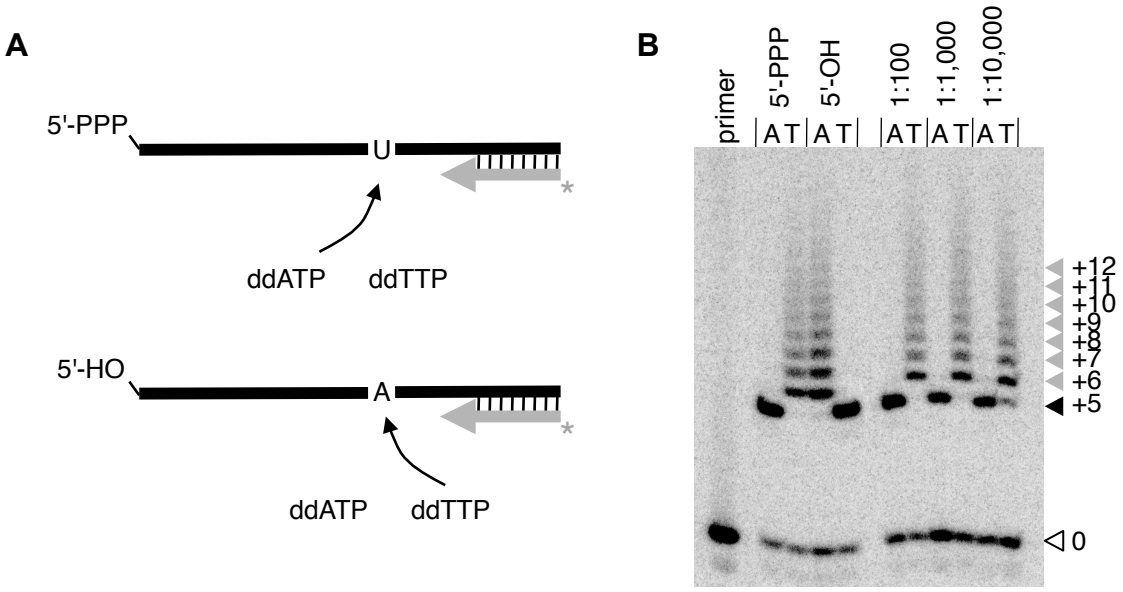

Figure S1. Estimation of the enrichment factor for one round of the selection. The assay was based on poisoned primer extension analysis of three mock-pools after one round of selection. **(A)** Schematic that shows 'active' (top) and 'inactive' RNA pool molecules. They are mutated with an A and a U five nucleotides upstream of the reverse transcription primer (grey arrow), which is 5'-radiolabeled (grey asterisk). During reverse transcription the primer extension on 'active' pool molecules is stopped at position +5 by ddATP but not by ddTTP. Conversely, the primer extension on the 'inactive' molecules is stopped at position +5 by the incorporation of ddTTP but not by ddATP. **(B)** Autoradiogram of denaturing PAGE-separated products from the poisoned primer extension assay. The "A" or "T" indicates whether ddATP or ddTTP was used in the reaction. The length of the extension of the primer is indicated on the right by an empty triangle for unelongated primers, by a black triangle for primers that were stopped by ddNTPs at position +5, and by grey triangles for primer extension products that were not stopped at position +5. Note that the templating sequence for longer extension products is the randomized region of the pool, which causes an exponential distribution of primers that were stopped at positions higher than +5. The behavior of the 'active' RNA (5'-PPP) and the 'inactive' RNA (5'-OH) is shown in the left half of the image. The behavior of pools that were enriched from dilutions of active in inactive pools at ratios of 1:100, 1:1,000, and 1:10,000, is shown in the six lanes on the right. Note that the band at +5 in the right-most lane shows that ~10% of the primer extensions were stopped by the incorporation of ddTTP, thereby detecting the presence of 10% A at position +5. This enrichment from 1:10,000 to ~ 0.9 in one selection step suggested a lower estimate of 9,000-fold for the enrichment of active sequences in one round of selection.

Figure S2

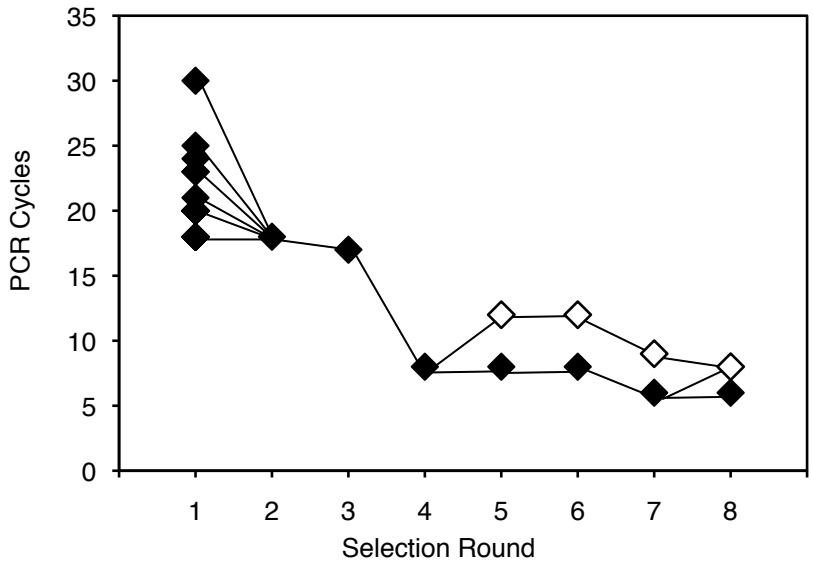

Figure S2. Progress of the in vitro selection. The graph shows the number of PCR cycles that was required in each round of the selection to obtain a band clearly visible by ethidium-bromide staining of agarose gels. The first round of selection was done in 11 parts, with PCR cycle numbers between 18 and 30. The drop in the PCR cycle number from >16 cycles (rounds one to three) to 8 cycles (round four) showed that a strongly increased amount of RNAs survived the selective step, suggesting that pool four contained a large fraction of active ribozymes. After round four, mutagenic PCR was employed to allow exploring the sequence neigborhood of selected clones, and the pool was split in two branches, one for low selection pressure (three hours incubation with trimetaphosphate; filled diamonds) and one for high selection pressure (five minutes incubation with trimetaphosphate; empty diamonds). After round seven, one additional branch was created, by subjecting a portion of the low-selection pressure branch to high selection pressure. Ten sequences were obtained from high- selection pressure and low selection pressure branches after five rounds and after eight rounds of selection. The name of the clone (e.g. R8_35C18) describes the round in which the clone was isolated (e.g. R8), the history of three-hour or five-minute incubations it experienced (e.g. _35), and the clone number (e.g. C18).

Figure S3

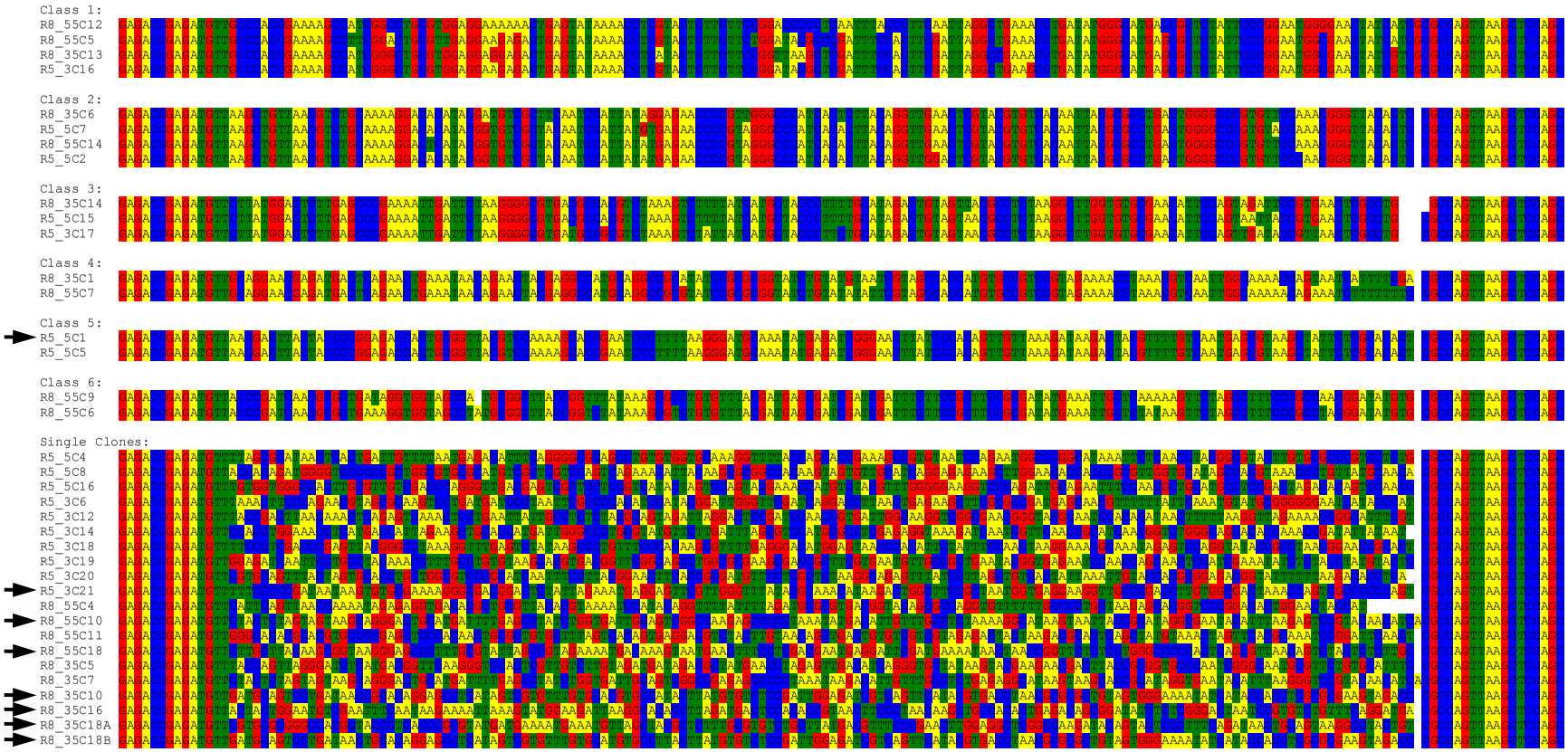

Figure S3. Sequences isolated after five or eight rounds of in vitro selection. Nucleotides are colored to facilitate comparison between related sequences and highlight the constant regions at the 5'- and 3'-ends. Six classes of sequences were isolated as multiple clones. Most clones were present as single clones. The eight most active clones are indicated with arrows. Note that the sequence of clone R5_5C1 is identical to the sequence of clone R5_5C5, therefore only one of the two clones was tested for activity.

Figure S4

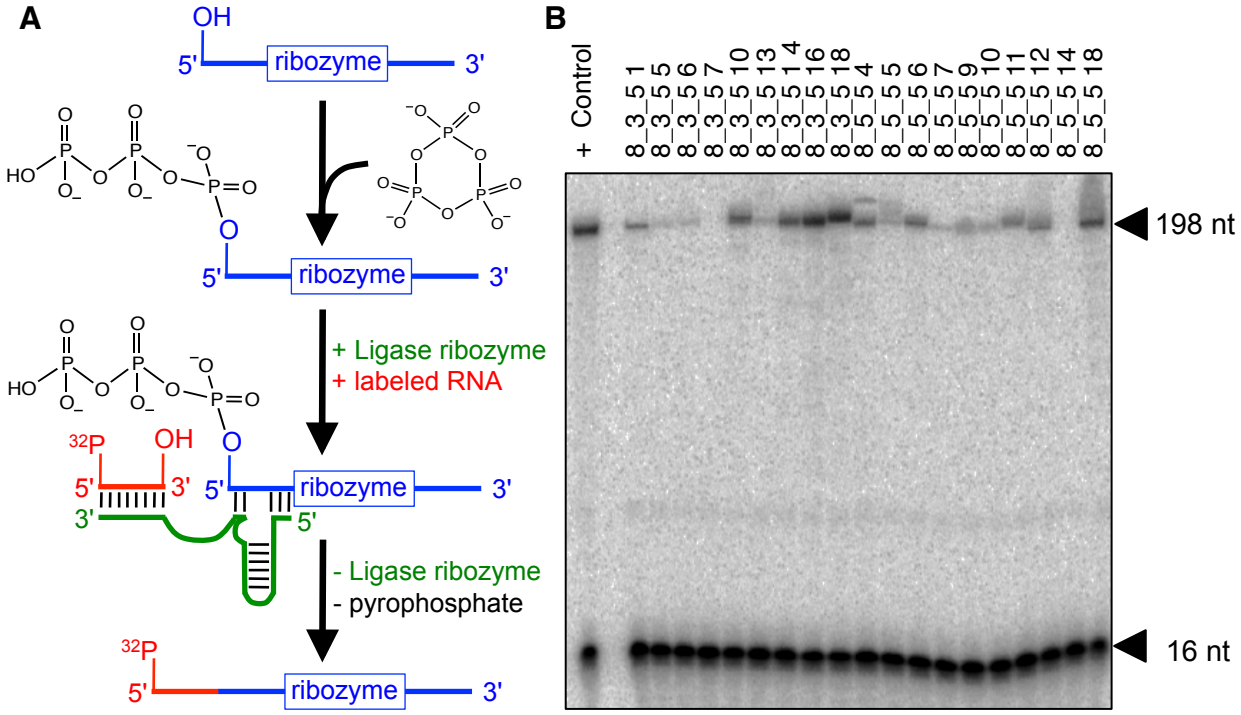

Figure S4. Assay for triphoshorylation activity using the R3C ligase ribozyme. (A) Schematic of the assay. After the selected RNA clone (blue; 182 nucleotides) was incubated with trimetaphosphate, ligase ribozyme (green; 16 nucleotides) and 5'-[^{32}P]-radiolabeled substrate RNA (red) were added, and incubated further. RNAs that contained a triphosphorylated 5'-terminus thereby generated a 198 nucleotide long product. (B) Autoradiogram of products from the ligase ribozyme reaction, after separation by denaturing 10% polyacrylamide gel electrophoresis. The labeled substrate RNA (16 nt) is shifted if the selected RNA clone carried a 5'-triphosphate, thereby generating a large gel shift (198 nt).

Figure S5

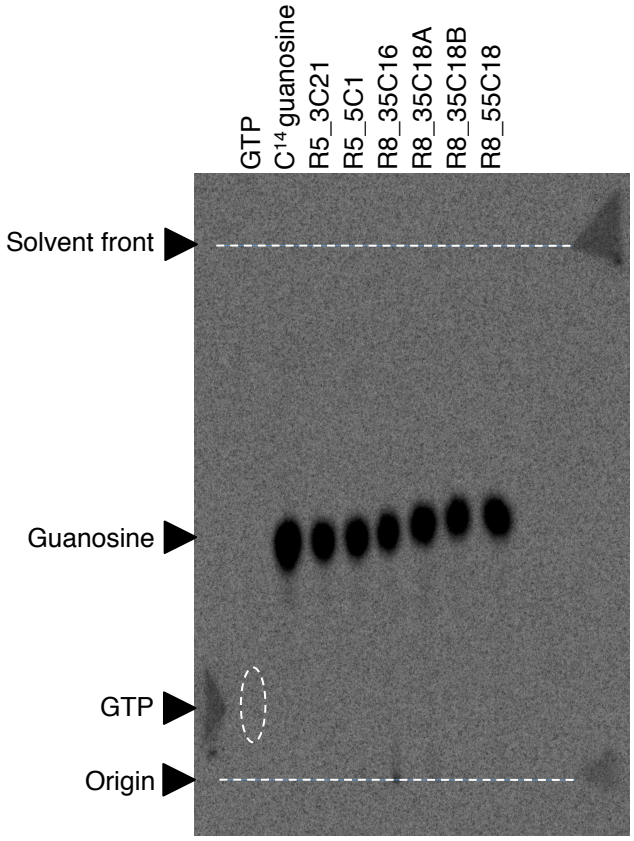

Figure S5. Assay testing for triphoshorylation of free nucleosides. The eight ribozymes that showed the highest activity in the self-triphosphorylation assay using the ligase ribozyme (figure 3) were tested. The 5'-terminal guanosine was removed from all ribozymes to allow for the insertion of a free guanosine. Ribozymes were incubated with C^{14} radiolabeled guanosine and trimetaphosphate. Standard reaction conditions were 8 µM ribozyme, 0.01 µCi C^{14} labeled guanosine, 100 mM $MgCl_2$, 50 mM trimetaphosphate, 50 mM Tris/HCl pH 8.3, and 16 hours incubation at room temperature. Variations used different reaction temperature (40°C, -20°C), and higher pH (9.5). 2 µL aliquots of the reactions were spotted on pre-wetted PEI cellulose, together with GTP as control. Plates were developed vertically with 1 M KH_2PO_4 (pH 3.5) and dried. GTP was visualized by UV shadowing. Radioactive signals were detected by phosphoimaging. Triangles that are weakly visible in the autoradiogram were used to mark the position of the origin, the solvent front, and the GTP. Only six of the eight tested ribozymes are shown here; the remaining two ribozymes showed the same negative result.